A novel function of tissue-type transglutaminase: protein disulphide isomerase

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We have found that tissue-type transglutaminase (tTG), also called TGc, TGase2 and $G\alpha_h$, has the activity of protein disulphide isomerase (PDI). We have shown that tTG converts completely reduced/denatured inactive RNase A molecule to the native active enzyme. The PDI activity of tTG was strongly inhibited by bacitracin, which is a frequently used inhibitor of conventional PDI activity. It was substantially inhibited by the simultaneous presence of other potential substrate proteins such as completely reduced BSA, but not by native BSA. This activity was especially high in the presence of GSSG, but not GSH. The addition of GSH to the reaction mixture in the presence of GSSG at a fixed concentration up to at least 200-fold excess did not very substantially inhibit the PDI activity. It is possible that tTG can exert PDI activity in a fairly reducing environment like cytosol, where most of tTG is found. It is quite obvious from the following observations that PDI activity of tTG is catalysed by a domain different from that used for the transglutaminase reaction. Although the alkylation of Cys residues in tTG completely abolished the transglutaminase activity, as was expected, it did not affect the PDI activity at all. This PDI activity did not require the presence of Ca^{2+}. It was not inhibited by nucleotides including GTP at all, unlike the other activity of tTG.

Key words: cytosol, disulphide, glutathione, nucleotide, ribonuclease A.

INTRODUCTION

Tissue-type transglutaminase (tTG) is unique among members of transglutaminase (TGase); it is known to have multiple functions, and its distribution is very diverse. The first well-known function is common among the members, which is the introduction of isopeptide linkages, $\varepsilon(\gamma$-glutamyl) lysine cross-links, within a single polypeptide or between different polypeptides [1]. The presence of Ca^{2+} is obligatory for this function. The second well-known function is the participation in signal-transduction pathways as $G\alpha_h$ [2,3]. It binds and hydrolyses GTP, and mediates the information transfer from agonist-bound receptors to downstream effectors such as phospholipase C (PLC) [4–7]; it makes a heterodimeric complex with a ≈ 50-kDa protein [8] and functions as a G-protein. It was demonstrated that tTG and PLCδ1 formed a complex and that PLC was inactive in this form, but that the binding of GTP to tTG in this complex induced the dissociation of PLC from the complex and that PLC became active [9]. On the other hand it was shown that the binding of GTP inhibits the formation of isopeptide cross-links, especially if the concentration of Ca^{2+} is low [10,11]. It was demonstrated that the binding of GTP and Ca^{2+} changed the tertiary structure of tTG to the opposite directions [12]. It was revealed by physical measurements that Ca^{2+} made the molecule more wide and open, and GTP made the molecule tighter and the exposure of the active site for TGase became more difficult. It was shown that the binding of GTP made tTG less susceptible to the degradation by proteases [10,13], probably due to the tightening of the tertiary structure of the molecule. As the intracellular environment is characterized by a relatively high concentration of GTP, in the millimolar region, and relatively low concentrations of Ca^{2+}, it is now generally believed that the intracellular tTG mainly

functions as $G\alpha_h$ instead of TGase [14]. It is, however, necessary for tTG to be somehow transported into the plasma membrane in order to function as a G-protein. The majority of tTG is found in cytosol [15], where tTG may not have any function. It was reported that rat brain astrocytes expressed two different isoforms of tTG, the long and the short forms, after treatment with cytokines such as interleukin-1β [16]. The short form apparently lacks the site for the interaction with GTP and the activity of TGase was much less inhibited by GTP. It was further reported that the brain of Alzheimer's disease patients contained a substantial proportion of the short form of tTG, and the formation of tau aggregation could be accounted for by the presence of this short isoform of tTG [17]. It was demonstrated that the expression of tTG was up-regulated by retinoic acid and apoptosis was induced [18,19], and that the apoptosis was prevented by the addition of the inhibitor of tTG [20]. The transfection of sense and antisense cDNA for tTG induced and suppressed the apoptosis, respectively [21]. This tTG-mediated apoptosis was reported to be relevant to certain kind of disease states such as AIDS [22]. However, a question still remains as to the physiological function of the normal isoform of tTG in the cells. As the distribution of tTG is so diverse, we thought that it might have a somewhat more fundamental activity than those two hitherto known functions. We started looking into the possibility that tTG might have the activity of protein disulphide isomerase (PDI) because of the presence of the active Cys in the molecule.

PDI is a typical resident protein of the lumen of the endoplasmic reticulum (ER) and a member of the thioredoxin superfamily [23–25]. The domain structure and various interesting functions of PDI family have been well documented in an excellent review article [25]. PDI introduces disulphide bridges at correct sites within polypeptides and contributes to constructing proper conformation for various proteins.

EXPERIMENTAL

Materials

Bacitracin, dithiothreitol (DTT) and iodoacetamide (IAA) were purchased from Nacalai Tesque (Kyoto, Japan). Sephadex G-25 and G-150 were purchased from Amersham Biosciences (Little Chalfont, Bucks., U.K.). RNase A and PDI were prepared from porcine pancreas and bovine liver, respectively, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and other reagents were purchased from Sigma (St. Louis, MO, U.S.A.).

Purification of tTG

tTG was purified from guinea-pig liver using ion-exchange chromatography on DE52 followed by immunoaffinity chromatography of an anti-tTG monoclonal antibody 8D, which was a kind gift from Dr K. Ikura (Kyoto Institute of Technology, Kyoto, Japan) [26].

Reduction and denaturation of proteins

Reduced RNase A (rdRNase) and BSA (rdBSA) were prepared by the method described previously [27]. Briefly, native proteins were dissolved (20 mg/ml) in 0.1 M Tris/HCl buffer (pH 8.6) containing 0.15 M DTT and 6 M guanidine/HCl. They were incubated at room temperature for 18 h. Proteins were then separated from small molecules by Sephadex G-25 column, which was equilibrated with 10 mM HCl or acetic acid, and they were stored at −80 °C until use. It was confirmed from the determination of free SH groups described below that almost all the disulphide bonds were reduced in those proteins.

Determination of PDI activity

PDI activity was determined by the measurement of the reactivation of rdRNase. Various amounts of tTG and rdRNase were incubated in 200 mM Tris/HCl buffer (pH 7.5) at 37 °C and aliquots were taken to measure the activity of RNase. Air/oxygen was removed from the reaction mixture as much as possible by replacing it with nitrogen gas, except in the experiments presented in Figures 5 and 7 (see below). The reaction tube was filled with the gas and tubes were sealed tightly during the incubation.

Determination of RNase activity

Yeast RNA was dissolved in 50 mM Tris/HCl buffer (pH 7.5) and was precipitated with ethanol to remove the unprecipitable fraction. Precipitated RNA was suspended in the same buffer, mixed with the above-mentioned rdRNase, and was incubated for 10 min at 37 °C. The final concentrations of RNase and RNA were 36 nM and 1 mg/ml, respectively, unless otherwise described. After the reaction RNA was precipitated with ethanol by leaving the reaction mixture at −20 °C overnight, and the absorption measured at 260 nm of the supernatant, which contained RNA molecules that had been degraded and become unprecipitable with ethanol.

Determination of free SH groups in proteins

The number of SH groups in proteins were determined with DTNB according to the method described in [28]. tTG was dissolved at 1.14 μM in 100 mM borate/NaOH buffer (pH 9.0) containing 8 M urea. DTNB was added to this solution at 38.8 μM, and the
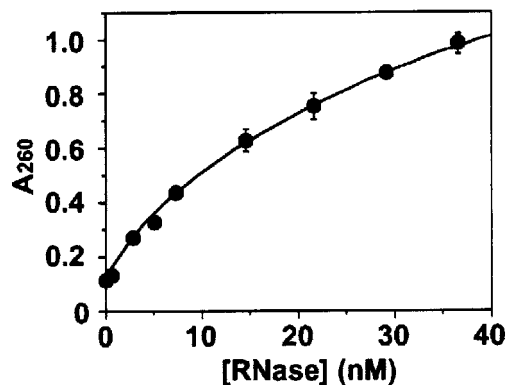

Figure 1   Dose-dependency of RNase reaction

RNase was incubated at varied concentrations with yeast RNA (1 mg/ml) at 37 °C for 10 min. After ethanol precipitation and centrifugation, absorbance at 260 nm of the supernatant was measured. The data shown are means ± S.E.M. from three independent experiments done in duplicate.

SH content of the protein was then assessed by measurement of the absorbance at 412 nm followed by the division by the molar absorption coefficient of the reduced form of DTNB.

Gel-filtration chromatography

tTG (1 mg) was loaded on to a Sephadex G-150 column (1.5 cm × 90 cm) equilibrated with 50 mM Tris/HCl buffer (pH 7.5), eluted with the same buffer (0.15 ml/min), and fractions of 1 ml were collected.

TGase activity assay

TGase activity of tTG was determined as described previously [29]. In brief, tTG was added to 20 mM Tris/HCl buffer (pH 7.4) containing 150 mM NaCl, 5 mM CaCl$_2$, 0.2 % acetylcasein and 50 μM dansylcadaverine, and then the increase of fluorescence intensity was monitored by fluorescence plate reader (CytoFluor Multi-well Plate Reader Series 4000; PerSeptive Biosystems, Framingham, MA, U.S.A.).

HPLC analysis of glutathione

Glutathione was analysed by HPLC using a Waters 625 LC System (Waters, Milford, MA, U.S.A.) equipped with a Resource RPC column (6.4 mm × 100 mm; Amersham Biosciences). The analysis was performed in isocratic mode using 1.6 % acetonitrile in water with 0.1 % trifluoroacetic acid. The flow rate was 1 ml/min, and the elution was monitored by absorption at 230 nm.

RESULTS

In order to find PDI activity of tTG, we used completely reduced RNase A as the substrate, except in one experiment shown in Figure 10 (see below), in which we used 'scrambled' RNase as the substrate. We determined the recovery of the RNase activity due to the formation of correct disulphide bonds by PDI activity. First of all we established the method for the determination of RNase activity using almost completely ethanol-precipitable RNA as the substrate. As is demonstrated in Figure 1,
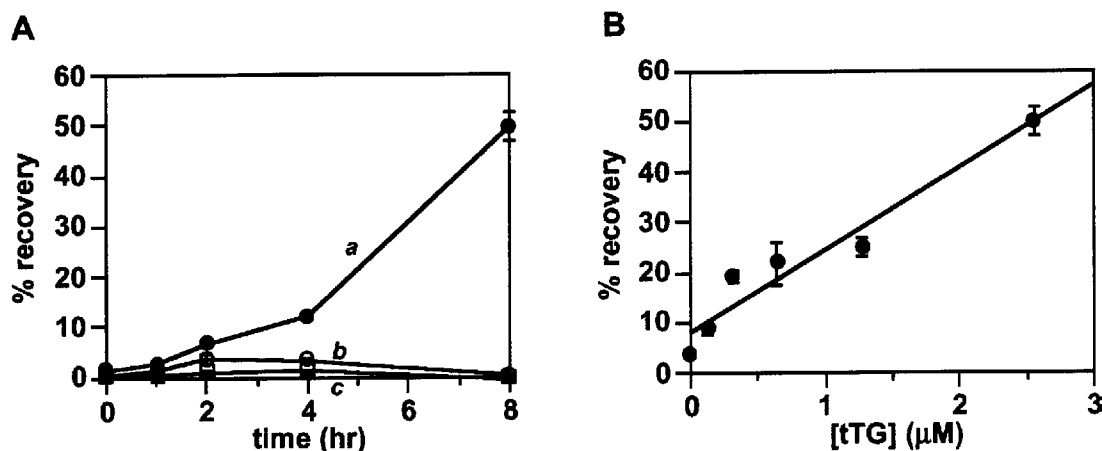
Figure 2   Reactivation of rdRNase with tTG

(**A**) rdRNase (2.9 μM) was incubated with (line a; ●) or without (line b; ○) tTG (2.5 μM) at 37 °C for different periods of time. The activity of RNase was measured as described in the Experimental section. The results obtained with tTG incubated without rdRNase (line c; □) are also shown. (**B**) rdRNase (2.9 μM) was incubated with tTG at increasing concentrations for 8 h at 37 °C, and the activity recovered was measured. The data shown are from one experiment that is a representative of at least three separate experiments. Vertical lines show the S.E.M. from duplicate determinations.

absorption at 260 nm in the supernatant increased in a dose-dependent manner with the concentration of RNase used, although in a non-linear fashion. It is obvious from this figure that absorption at 260 nm in the supernatant increases from about 0.1 to 1.0 after 10 min of incubation if one uses 36 nM RNase A, which is the standard concentration of the enzyme used throughout the present study. Using this figure as the standard curve one can easily calculate the percentage recovery of the activity of rdRNase, which is the measure of PDI activity. After the incubation with tTG, rdRNase at 36 nM was used for the determination of RNase activity. The rdRNase of course did not have any activity to increase this absorption at all.

Using this reliable method we looked at the effect of tTG on the recovery of the activity of rdRNase. As shown in Figure 2(A), the addition of tTG to rdRNase (line a) did increase with time the percentage recovery of RNase activity relative to that obtained by the incubation of rdRNase alone (line b). tTG itself, however, did not degrade RNA at all (Figure 2A, line c). Although the self-recovery of rdRNase was not negligible, the PDI activity of tTG was very obvious. Figure 2(B) shows the dose-dependency of tTG on the PDI activity. By incubating a fixed amount of rdRNase with tTG at varied concentrations for 8 h the percentage recovery of the activity of rdRNase steadily increased.

According to previous works on PDI, it is generally believed that free SH groups play important roles in the catalysis [25]. Having this notion in our minds, we decided to look into the importance of SH groups in tTG-mediated PDI activity. It has been well established that tTG has a Cys residue at position 277 as the active centre for the TGase activity [30,31], which is the introduction of isopeptide cross-links between protein-bound Gln residues and small primary amine molecules. The alkylation of $92.7 \pm 4.6\%$ of the free SH groups including this residue with IAA, which was determined by the method described in the Experimental section, readily abolished the TGase activity, as is shown in the inset of Figure 3. To our surprise, however, it did not affect the PDI activity at all, as is clearly demonstrated in Figure 3 (compare columns 1 and 2). It became evident that the active sites for these two activities were different from each other. The oxidation of rdRNase shown in Figure 2 is puzzling, as there is almost no oxygen and no oxidants. Where are the oxidizing equivalents coming from? We believe that the disulphide bonds,
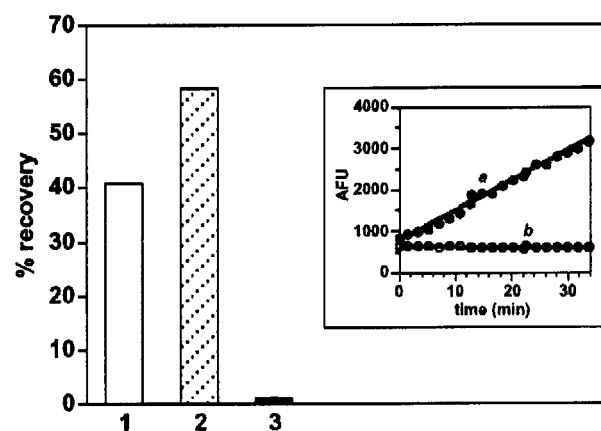
Figure 3   Differential effects of modification of Cys in tTG on the activity of PDI and TGase

tTG (5.1 μM) was alkylated with 1 mM IAA for 1 h under denaturing conditions in the presence of 3.2 M urea, and then dialysed against 50 mM Tris/HCl buffer (pH 7.5). TGase activity was determined using tTG (2.5 μM) with (line b) and without (line a) the modification. The results are shown in the inset. PDI activity was also measured using the substrate rdRNase (14.7 μM) and tTG (2.5 μM) with (column 2) and without (column 1) the modification. The IAA-treated tTG was reduced further with DTT and then alkylated with IAA. The PDI activity of this preparation was similarly determined after extensive dialysis to remove these reagents (column 3). The reaction was carried out at 37 °C for 8 h. The data shown are from one experiment that is representative of at least three separate experiments. Vertical lines show the S.E.M. from duplicate determinations.

which are probably present in tTG, may play a crucial role. To confirm this notion we have reduced the above-mentioned alkylated tTG by DTT and further alkylated the newly formed free SH groups with IAA, if there is any. The result is demonstrated in Figure 3 (column 3). It is quite obvious that the further alkylation now completely abolished the activity of tTG-mediated PDI activity. This procedure should not affect the PDI activity at all unless there are disulphide bonds present in the tTG molecule, and it has become obvious that there are. Whether these disulphide bonds are actually involved in the PDI reaction remains to be investigated further.
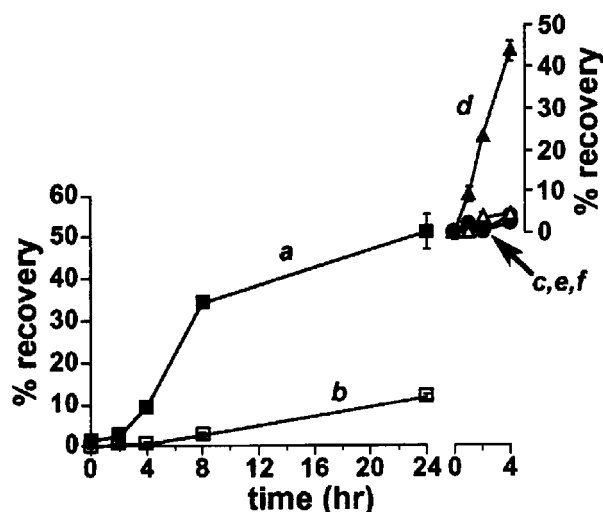
Figure 4   Reduction and re-oxidation of disulphides in tTG during the PDI reaction

The IAA-treated tTG (2.5 μM) was incubated with rdRNase (2.9 μM) for up to 24 h at 37 °C, and the activity of RNase was measured at different time points during this incubation (line a; ■). The self-renaturation of rdRNase (line b; □) was also determined. After 24 h the proteins in the reaction mixture were alkylated with 1 mM IAA for 1 h in the presence of 4 M urea, and then dialysed against 50 mM Tris/HCl buffer (pH 7.5). PDI activity of tTG (0.25 μM) with (line c; ●) or without (line d; ▲) the modification was measured by the addition of a new batch of rdRNase (1.5 μM), GSH (0.5 mM) and GSSG (10 μM). The values of percentage recovery due to the self-renaturation of rdRNase with (line e; ○) or without (line f; △) the modification were also shown. The data shown are from one experiment representative of at least three separate experiments. Vertical lines show the S.E.M. from duplicate determinations.

We have shown in Figure 3 that disulphide bonds might be involved in tTG-mediated PDI activity. However in those experiments, disulphide bonds present in the enzyme may only be reduced to become free SH groups due to the oxidation of the substrate and may never be re-oxidized to become disulphides. If this is the case, the alkylation of the newly formed SH groups with IAA should abolish the PDI activity even in the presence of oxidants such as GSSG. The newly formed SH groups should be re-oxidized to disulphides, which may play an important role for tTG to carry out another cycle of catalysis. In order to clarify this point, we have used IAA-treated tTG and carried out the PDI reaction for some time in the absence of oxidants (Figure 4, line a). We then added IAA to the reaction mixture to alkylate newly formed SH groups during the reaction. After extensive dialysis to remove IAA, the PDI activity of tTG was investigated by the addition of a new batch of rdRNase substrate and GSSG + GSH. As is demonstrated by Figure 4 (line c), there was no activity of PDI detected after the alkylation of the enzyme with IAA. We have also added GSSG + GSH to the reaction mixture without the treatment with IAA. In this case, the PDI reaction, which had been slowed down, probably due to the reduction of disulphides, became quite obviously faster (Figure 4, line d). This result strongly implies that disulphide bonds are acting as oxidizing equivalents in tTG-mediated PDI reaction. It remains to be elucidated in the future which disulphide bonds are involved in this catalysis.

We then decided to actually determine the number of disulphide bonds in the tTG molecule by the method described in the Experimental section. First of all, we determined the protein concentration of the tTG preparation by BCA protein assay kit (Pierce, Rockford, IL, U.S.A.) using BSA as the standard protein. The molar concentration was then obtained by dividing this value by

77010, which is the molecular mass of tTG deduced from its primary structure [32]. The preparation of tTG was then mixed with DTNB in the presence of 8 M urea. The number of free SH groups in the molecule was determined by dividing the absorption at 412 nm by the molar absorption coefficient, $14\,290\ \mathrm{M^{-1} \cdot cm^{-1}}$, of the reduced form of DTNB [28], followed by division by the molar concentration of the tTG preparation obtained above. From three independent determinations, we obtained values of 5.13–7.32. As the total number of Cys residues in guinea-pig liver tTG is known to be 17 [32], the number of disulphide bonds in the tTG preparation would be five or six.

We purified tTG from guinea-pig liver extract by affinity chromatography of a monoclonal antibody against tTG, and the purity of the final preparation was very high, as demonstrated in the inset of Figure 5(A). It is still possible, although it is quite unlikely, that a contaminating minor component(s) but not tTG itself actually has the PDI activity. In order to rule out this remote possibility we performed the following experiments (shown in Figure 5). We placed the purified tTG on a Sephadex G-150 column to carry out gel-filtration chromatography. The major peak had an apparent molecular mass of about 78 kDa, as was expected. The protein in the peak fraction of this chromatogram showed a very clear sharp band of about 73 kDa upon SDS/PAGE under non-reducing conditions. The protein in this fraction and that purified by the affinity chromatography showed very comparable specific activity, as shown in Figure 5(B). It is now very safe to conclude that tTG itself has PDI activity.

The antibiotic bacitracin has been used fairly commonly as an inhibitor of PDI activity in the past [33,34]. We used it to see whether it also inhibits this newly identified activity of tTG. As Figure 6 (line a) shows, it almost completely inhibited the tTG-mediated reactivation of rdRNase at 1 mM. We looked at the effect of this compound on the activity of TGase to examine the specificity. Although inhibition was observed, the reason for this inhibition is actually understandable; the molecule has free amino groups, which should strongly inhibit TGase activity in our assay system. We further investigated the effect of putrescine, which is a strong inhibitor of TGase, on tTG-mediated reactivation of rdRNase. This amino-group-containing compound did not inhibit the tTG-mediated reactivation of rdRNase (Figure 6, line b). Neither bacitracin nor putrescine inhibited the activity of RNase itself (Figure 6, points c and d). These results would strengthen the notion that the mechanism of tTG-mediated reactivation of rdRNase is similar to that of conventional PDI.

We then studied the effect of other proteins on the tTG-mediated reactivation of rdRNase, assuming that the simultaneously added proteins with free SH groups should interfere with the activity, providing that what we are looking at is actually PDI activity. This is because the substrate specificity of PDI is usually quite broad. To this end we employed rdBSA and native BSA. Figure 7(A) shows clearly that the former inhibited the activity very strongly (line b) but the latter did not at all (line a). The inhibition was dependent on the concentration of rdBSA added, keeping the concentration of the substrate rdRNase constant. As the effect was specific for the reduced form of BSA, it looks as if these two proteins, rdRNase and rdBSA, compete as the substrate for the PDI activity of tTG. This result could also be explained if we assume that rdBSA and rdRNase make complexes, and that the latter becomes unable to function as an RNase. In order to clarify this possibility, SDS/PAGE was performed under non-reducing conditions. As shown clearly in Figure 7(B), the amount of rdRNase did not decrease due to the formation of complex with rdBSA. rdRNase is, therefore, still available as the substrate for the PDI activity. In fact, any aggregate of these two proteins, either alone or in combination, was not observed.
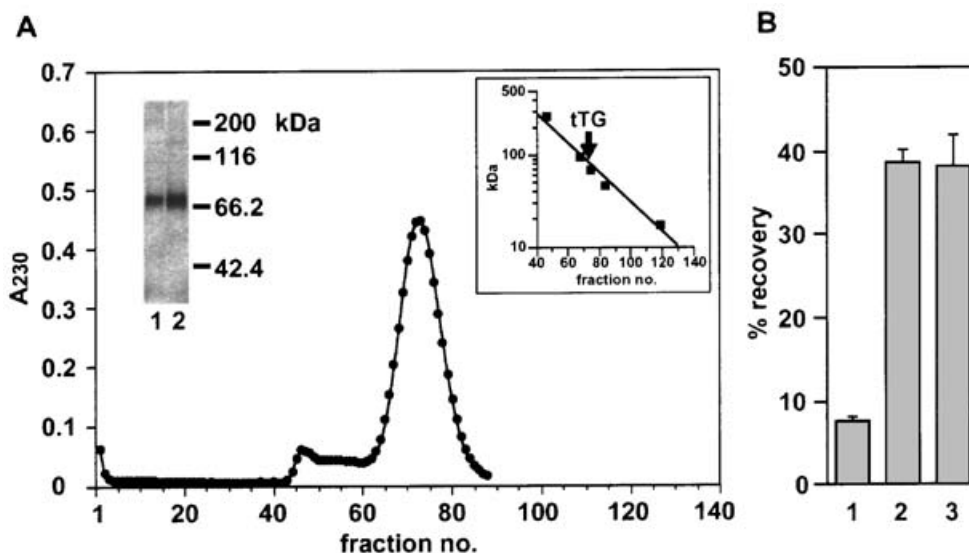
Figure 5   Gel-filtration chromatography of tTG

(**A**) Gel filtration of tTG. tTG (1 mg) was loaded on to a Sephadex G-150 column. Fractions (1 ml) were collected, and then absorption at 230 nm was measured (●). The calibration curve obtained using catalase (250 kDa), plasminogen (91 kDa), BSA (66 kDa), ovalbumin (45 kDa) and lysozyme (16.5 kDa) as molecular-mass markers is shown in the right-hand insert (■). The results of SDS/PAGE (7.5 % gel) analysis done under non-reducing conditions using tTG before gel filtration (lane 1) and fraction no. 73 (lane 2) are shown in the left-hand insert. (**B**) A comparison of specific activity of PDI before and after gel-filtration chromatography. rdRNase (14.7 μM) was incubated with the affinity-purified tTG (2.5 μM, lane 2) or fraction no. 73 (2.5 μM, lane 3) in the presence of 10 μM GSSG for 1 h at 37 °C, and the recovered RNase activity was measured. The self-renaturation of rdRNase was shown in lane 1. In this experiment we did not remove air from the experimental system. The data shown are from one experiment that is representative of at least three separate experiments. Vertical lines show the S.E.M. from duplicate determinations.

Figure 6   Bacitracin inhibits reactivation of rdRNase by tTG

rdRNase (14.7 μM) was incubated with tTG (2.5 μM) for 8 h at 37 °C in the presence of bacitracin (line a; ●) or putrescine (line b; ○) at varied concentrations. Native RNase (14.7 μM) was also incubated in the presence of bacitracin (line c; ■) or putrescine (line d; □) at 1 mM. The data shown are from one experiment that is representative of at least three separate experiments. Vertical lines show the S.E.M. from duplicate determinations.
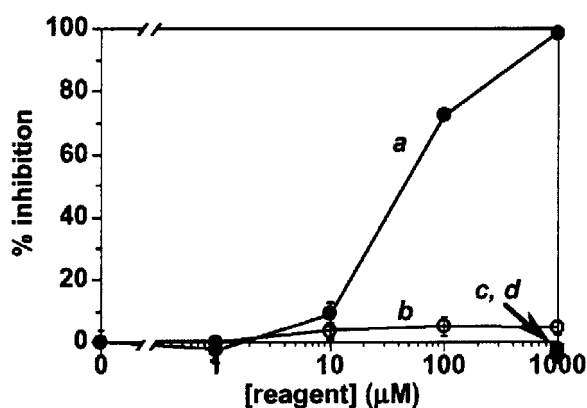
We then investigated the effect of glutathione on the tTG-mediated reactivation of rdRNase. Figures 8(A) and 8(B) demonstrate the effect of GSH and GSSG, respectively, on the activity. It is quite clear that GSSG accelerated the activity, but that GSH did not. This result was anticipated somewhat, as the reaction is essentially an oxidation reaction. It is of interest to see how the activity supported by GSSG is inhibited or activated by GSH. We fixed the concentration of GSSG at 10 μM and added GSH at varied concentrations. As is very clearly depicted in Figure 8(C), GSH accelerated but did not substantially inhibit the GSSG-supported activity until it was added at 2 mM. The ratio of GSH to

GSSG then was 200:1. This acceleration by GSH at not very high concentrations is understandable, as the PDI-catalysed reaction consists of not only oxidation but also reduction/isomerization of disulphides. The inhibition by GSH could be expected to become weak if GSH is oxidized to GSSG during the reaction. In order to check this possibility we determined the purity of GSH after it was incubated in the same way as in the actual experiment. According to the result of the HPLC analysis (results not shown), there was no obvious or substantial oxidation of GSH to GSSG during the incubation. It is not necessary, therefore, to consider the oxidation of added GSH to GSSG under the experimental conditions.

We next tried to show more clearly the enzyme-catalysed 'turn-over' of the PDI reaction by carrying out the reaction in the presence of GSSG + GSH and much lower amounts of the enzyme and substrate. The concentrations of tTG, rdRNase and GSSG were 26 nM, 370 nM and 10 μM, respectively. There are four disulphide bonds required for rdRNase to become fully active. As is depicted clearly in Figure 9 by the difference between lines a and c, about 20 % of the activity of rdRNase was recovered after 6 h of incubation under these experimental conditions. To recover 20 % of the activity of rdRNase, it may be possible to think that the number of disulphides formed in the substrate is much more than that reduced in the enzyme. It would be necessary for us to assume that the disulphides present in tTG should be reduced cyclically and re-oxidized to carry out multiple catalysis reactions. This turning over of the PDI reaction might have become possible only in the presence of GSSG. In fact, the reaction carried out in the absence of GSSG shown by the difference between lines b and d in Figure 9 does not seem to have proceeded obviously. It is understandable and indeed expected if one compares the concentration of the enzyme (26 nM) with that of the substrate (370 nM).

Thus far we had used rdRNase exclusively as the substrate for PDI activity measurement. We think that not only simple
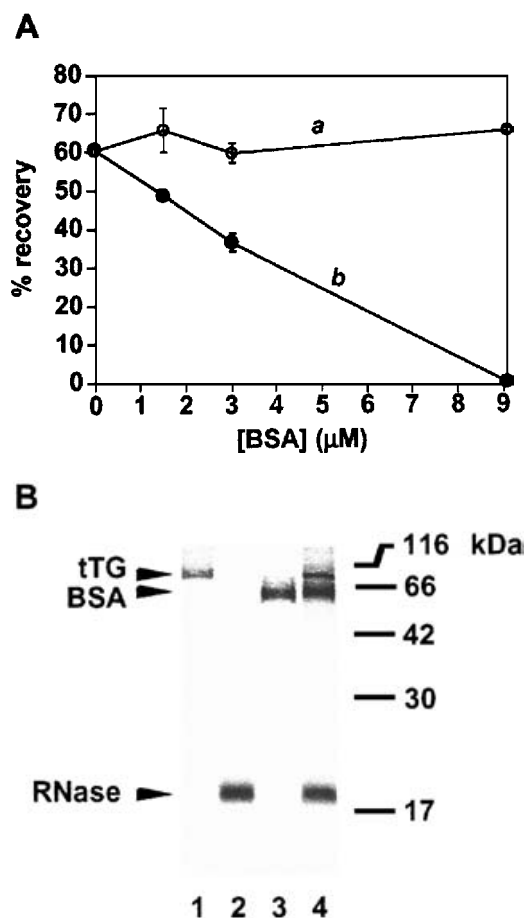
Figure 7   Reactivation of rdRNase by tTG is inhibited by rdBSA but not by native BSA

(**A**) rdRNase (14.7 μM) was incubated with tTG (2.5 μM) for 12 h at 37 °C in the presence of native BSA (line a; ○) or rdBSA (line b; ●) at varied concentrations. In this experiment we did not try to remove air from the reaction mixture. The data shown are from one experiment representing at least three separate experiments. Vertical lines show the S.E.M. from duplicate determinations. (**B**) SDS/PAGE (13 % gel) was performed under non-reducing conditions to investigate the interaction between rdRNase and rdBSA during the incubation with tTG. rdRNase (14.7 μM) was incubated with tTG (2.5 μM) in the presence of rdBSA (9.1 μM) at 37 °C for 12 h. An aliquot was subjected to SDS/PAGE (lane 4). Equivalent amounts of each ingredient, tTG (lane 1), rdRNase (lane 2) and rdBSA (lane 3) was also loaded separately.

oxidation but also reduction/isomerization have to be involved to convert the inactive rdRNase back to the active RNase. We do not believe that disulphide bonds are always made in the substrate at the right positions immediately and that there is no need to reduce/isomerize the bonds to place them in the right positions. To further substantiate the notion that the tTG-mediated reaction is actually the PDI reaction, however, we decided to use 'scrambled' RNase as the substrate in this particular experiment. With this substrate it is obvious that disulphide bonds have to be reduced and isomerized for the molecule to become the active enzyme with disulphide bonds in the correct positions. It is shown in Figure 10 that tTG can not only oxidize the SH groups in rdRNase but also reduce/isomerize disulphides in scrambled RNase. It is probably safe to conclude that it catalyses the *bona fide* PDI reaction.

The next obvious question that one might ask is how strong this newly identified activity of tTG-mediated PDI is relative to that of the conventional PDI. The results are demonstrated in Figure 11.
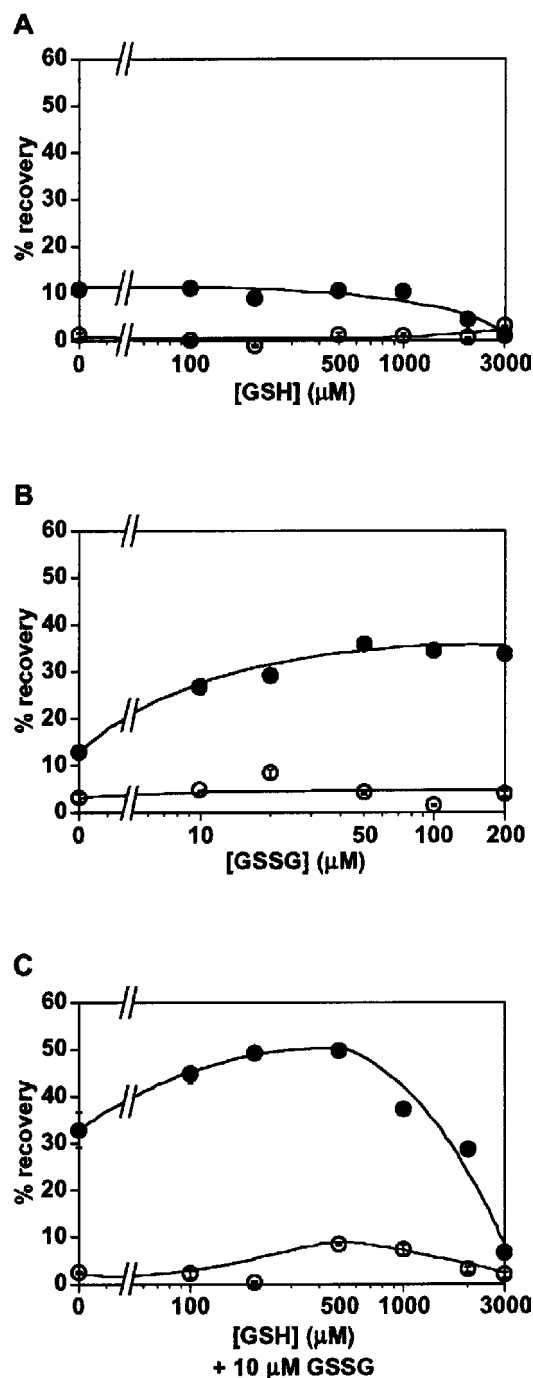

Figure 8   Effect of glutathione on the tTG-catalysed reactivation of rdRNase

rdRNase (2.9 μM) was incubated at 37 °C for 3.5 h in the presence (●) or absence (○) of tTG (2.5 μM) with (**A**) GSH at increasing concentrations, (**B**) GSSG at increasing concentrations and (**C**) 10 μM GSSG and GSH at increasing concentrations. The activity of RNase recovered was measured using 9 nM as the final concentration. The data shown are from one experiment representative of at least three separate experiments. Vertical lines show the S.E.M. from duplicate determinations.

Using a 10-fold greater concentration, tTG had only about 50 % of the activity of PDI. It is reasonable to conclude that the activity of tTG is about 5 % relative to that of the PDI under these experimental conditions. In experiments shown in Figures 8 and 11, in which glutathione was used, the concentration of rdRNase during the incubation with tTG was reduced from 14.7 to 2.9 μM
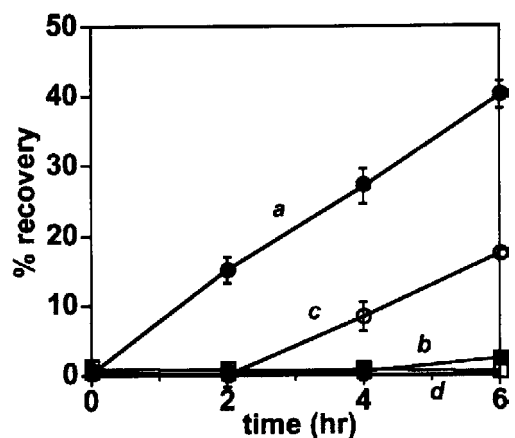
Figure 9   tTG can 'turn-over' the PDI reaction depending on glutathione

rdRNase (370 nM) was incubated with tTG (26 nM) in the presence (line a; ●) or absence (line b; ■) of GSH (0.5 mM) and GSSG (10 μM) at 37 °C for different periods of time, and the activity of RNase was measured. The values of percentage recovery due to the self-renaturation of rdRNase in the presence (line c; ○) or absence (line d; □) of GSH (0.5 mM) and GSSG (10 μM) were also shown. The data shown are from one experiment representative of at least three separate experiments. Vertical lines show the S.E.M. from duplicate determinations.
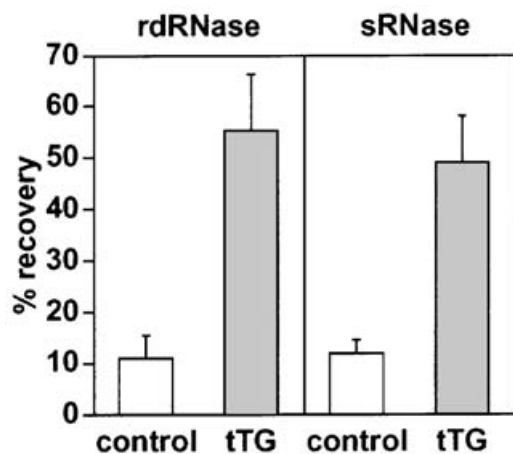

Figure 11   Comparison of the PDI activity of tTG and a commercial bovine liver PDI

rdRNase (2.9 μM) was incubated with either tTG (2.5 μM; line a; ●) or with a commercial bovine liver PDI (0.25 μM; line b; ▲) in the presence of 1 mM GSH and 10 μM GSSG at 37 °C for different periods of time. The recovered activity of RNase was measured using 9 nM as the final concentration. The control experiments, self-renaturation of rdRNase, were also performed without pre-incubation with tTG (line c; ○). The data shown are from one experiment that is a representative of at least three separate experiments. Vertical lines show the S.E.M. from duplicate determinations.

Figure 10   tTG reactivates not only rdRNase but also scrambled RNase

We dissolved the rdRNase (0.29 mM) in 150 mM Tris/HCl (pH 7.5) containing 6 M guanidine/HCl and incubated with rotation at room temperature for 1 week to make scrambled RNase (sRNase). The completion of the reaction was confirmed by the determination of SH with DTNB. The SH content was < 10 %. sRNase or rdRNase (2.9 μM) was then incubated with tTG (2.5 μM) at 37 °C for 3 h in the presence of GSH (1 mM) and GSSG (10 μM), and the activity of RNase was measured (shaded columns). The self-renaturation of sRNase or rdRNase is also indicated (white columns). The data shown are means from duplicate determinations of three separate experiments. Vertical lines show the S.E.M. from those six determinations.

to reduce the self-renaturation of rdRNase. It was also reduced during the incubation with RNA from 36 to 9 nM.

Having shown very clearly in Figure 3 that activities of PDI and TGase are mediated by different active sites, we then decided to investigate the effect of Ca^{2+} on the newly identified tTG-mediated PDI activity. It is a well-established fact that Ca^{2+} is obligatory for TGase activity. As demonstrated in Figure 12(A), the native tTG did not show the PDI activity in the presence of Ca^{2+}; it looked at first as if Ca^{2+} inhibited it. The reason for this apparent inhibition became obvious after SDS/PAGE analysis under reducing conditions (Figure 12B). As the polymers of
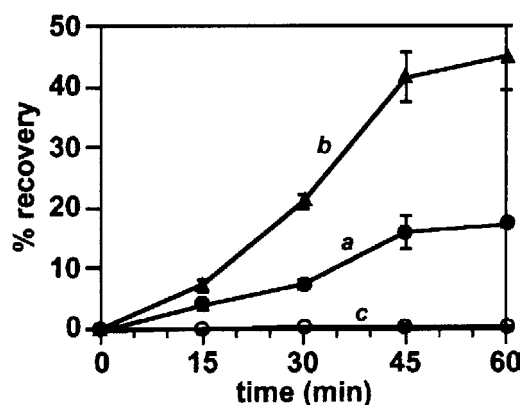
rdRNase did not dissociate into monomers under reducing conditions, it is evident that they were not polymerized by disulphide bonds but by isopeptide cross-links. The substrate rdRNase was polymerized due to the TGase activity of native tTG and it escaped being used as the substrate for PDI. Taking advantage of the result of the alkylation experiment, we then further studied the effect of Ca^{2+} using tTG alkylated with IAA. It became very clear that the PDI activity did not require Ca^{2+} at all, as shown in Figure 12(A). The inclusion of 10 mM EGTA in addition to 0.5 mM Ca^{2+} did not affect the PDI activity. TGase activity was inhibited completely under these conditions.

Finally we investigated the effects of various nucleotides on the PDI activity of tTG. As was expected, in the presence of 0.5 mM Ca^{2+} various nucleotides at 1 mM inhibited the TGase activity very strongly (Figure 13A). On the other hand, as is shown in Figure 13(B), the addition of nucleotides had no effect on PDI activity. As it is known that tTG has GTPase activity, we determined the hydrolysis of GTP to GDP and GMP using ion-pair HPLC [35]. Only about 10 % of GTP was converted to GDP in 12 h under the conditions used in this experiment (Figure 13B). The lack of inhibition of GTP on PDI activity could not be explained by the hydrolysis of GTP. In fact, GDP itself was just as effective as GTP in terms of the inhibition of TGase activity (Figure 13A).

DISCUSSION

The initial thing we had to deal with in this study was the choice of the substrate for the PDI activity. Many studies have used scrambled RNase as the substrate, which is made by re-oxidation of rdRNase in air in the presence of protein denaturants. It contains disulphide cross-links in positions different from the native molecule and is devoid of the enzyme activity. We decided not to use this scrambled RNase, except in one experiment shown in Figure 10, and chose the completely reduced RNase, as we doubted the quantitative reproducibility of the results due to the uncertain nature of the structure of the scrambled RNase. The next
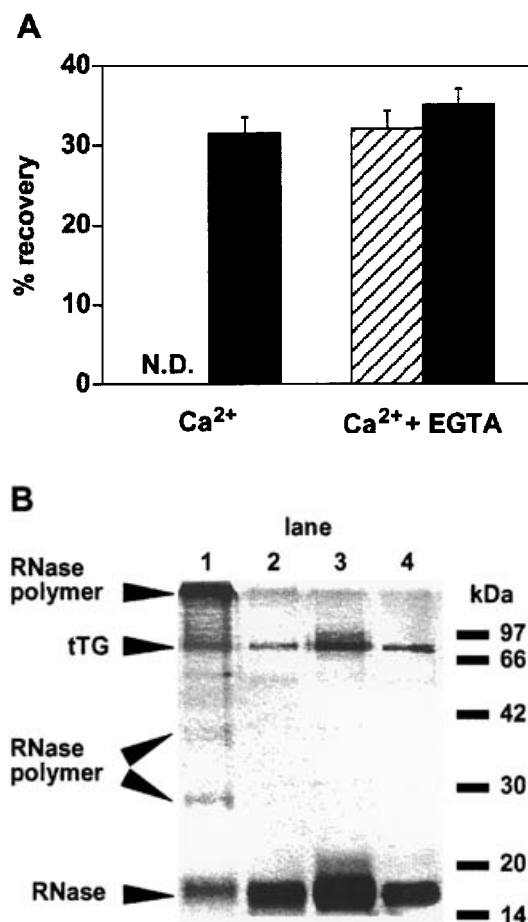
A

B

Figure 12   Effect of calcium on PDI activity of tTG

(**A**) PDI activity of native tTG (2.5 μM; hatched columns) and IAA-treated tTG (2.5 μM; shaded columns) was measured by incubating them with 14.7 μM rdRNase as the substrate at 37 °C for 12 h in the presence of 0.5 mM CaCl$_2$ and 10 mM EGTA. The PDI activity of the native tTG was not detected (N.D.) in the presence of CaCl$_2$ alone. The method for the IAA treatment of tTG was described in the legend for Figure 3. The data shown are from one experiment representative of at least three separate experiments. Vertical lines show the S.E.M. from duplicate determinations. (**B**) rdRNase (14.7 μM) was incubated with native tTG (2.5 μM; lane 1) or IAA-treated tTG (2.5 μM; lane 2) in the presence of 0.5 mM CaCl$_2$ at 37 °C for 12 h, and then applied to SDS/PAGE (13 % gel) analysis performed under reducing conditions. rdRNase was also incubated with native tTG (lane 3) or IAA-treated tTG (lane 4) in the presence of 0.5 mM CaCl$_2$ and 10 mM EGTA.

A

B

Figure 13   Nucleotides do not inhibit PDI activity of tTG

(**A**) TGase activity of tTG (2.5 μM) was determined in the presence of 0.5 mM CaCl$_2$ and 1 mM GTP (line b; ○), GDP (line c; ▲) or ATP (line d; ×). TGase activity of tTG without nucleotide was also shown (line a; ●). (**B**) PDI activity of tTG (2.5 μM; shaded columns) was determined in the presence of various nucleotides (1 mM) by incubating it with the substrate rdRNase (14.7 μM) for 12 h at 37 °C. The self-renaturation of rdRNase (hatched columns) was also determined. The data shown are from one experiment representative of at least three separate experiments. Vertical lines show the S.E.M. from duplicate determinations.

thing was the establishment of a reliable method for the determination of RNase activity. Most people make use of the fact that RNase can hydrolyse the phosphodiester bond in cytidine 2′,3′-cyclic monophosphate [36]. Although absorbance at 284 nm increases due to the hydrolysis, the substrate itself has very high absorption at this wavelength and the increment of the absorbance caused by the hydrolysis is almost negligible. We prepared almost completely ethanol-precipitatable yeast RNA by removing ethanol-soluble small molecules by centrifugation after precipitation with ethanol. Using this as the substrate, we could measure the substantial increase of absorbance at 260 nm from almost negligible background (Figure 1). Knowing the initial concentration of rdRNase, we could easily calculate the percentage recovery of RNase activity using this figure as the standard curve. It is difficult to say, however, what the percentage recovery really means. It could mean either the percentage of rdRNase that has all the correct disulphide bonds, or all the molecules that still have incorrect disulphide bonds with low activity
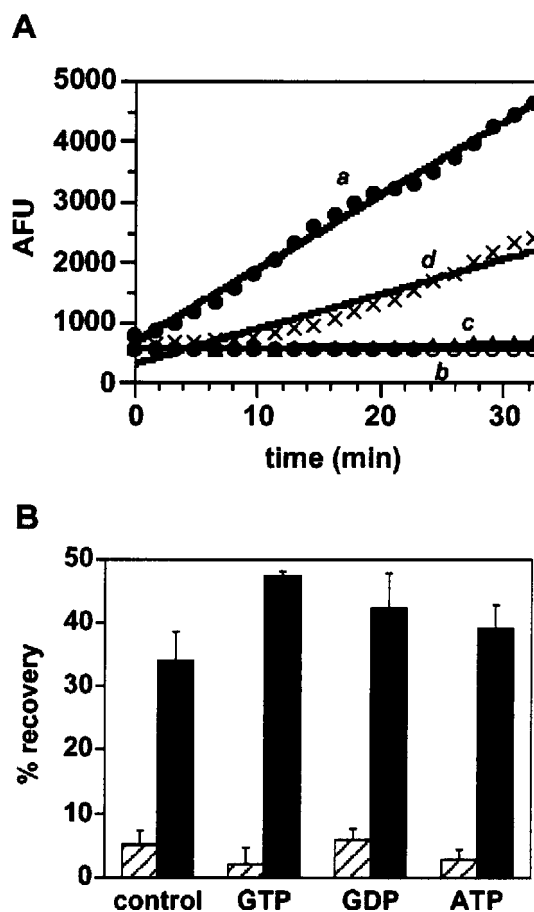
relative to the native molecule. Or else it could be more complicated than these two cases. At least we do not believe that there is a simple proportional relationship between the RNase activity and the number of disulphide bonds in the molecules. RNase has been the preferred substrate for PDI activity measurement, as it is convenient to determine the enzyme activity regained due to the PDI activity. One inherent problem associated with this substrate is self-reactivation. To reduce this as much as possible, we have tried to reduce the air/oxygen concentration in the reaction mixture by replacing it with nitrogen gas and tightly sealing the reaction tubes, except in experiments shown in Figures 5 and 7. Self-reactivation was still not completely suppressed (Figure 2, line b), but it became insubstantial. tTG itself had no activity to degrade RNA (Figure 2, line c). The difference between lines a and b in Figure 2 represents the PDI activity of the tTG preparation. In the experiment presented in Figure 2, the concentrations of the substrate rdRNase and the enzyme tTG were 2.9 and 2.5 μM, respectively. We do not know how many of the SH groups in rdRNase were oxidized and how many disulphides in tTG were reduced. We are in the process of investigating these points, and the results should be reported soon. Unlike conventional PDI, free SH groups normally present in the

molecule do not play any important role in tTG, as was shown in Figure 3. For disulphides to act as oxidizing equivalents, they ought to have special structure, which we do not know yet. Although native BSA has 17 disulphides, it never acted as a PDI, as is demonstrated clearly in Figure 7.

It is generally believed that proteins found in cytosol do not have disulphide bonds due to the reducing nature of the environment. However, several cytoplasmic proteins have been reported to have disulphide bonds. It was shown that glutathione reductase from human erythrocytes contained inter- and intra-polypeptide disulphides [37]. Protein disulphide-bond formation in cytosol has also been demonstrated to be essential for the maturation of vaccinia virus [38] and simian virus 40 [39] in host mammalian cells. Recently it has been reported that hyperthermophilic Archaea contain intracellular proteins with disulphide bonds [40]. However, it remains to be elucidated whether the intracellular redox status is quite different in these organisms from other living cells. It is of interest to note that one of those archaeal proteins found to have disulphides, adenylosuccinate lyase, was expressed in *Escherichia coli* and that the expressed protein had disulphides [41]. tTG is a typical protein found in cytosol, and it has been reported that it does not contain any disulphide bonds [42,43]. However, careful reading of the articles reveals that the authors used a higher molecular mass than the presently accepted value as the relative molecular mass of tTG and a slightly lower molar absorption coefficient for the reduced form of DTNB. The number of SH groups in tTG estimated using these numbers would inevitably be higher than the actual value. Furthermore, the number of free SH groups reported was quite different depending on the reagent used. It could be safer to say that the numbers of free SH and disulphide bonds in tTG have not yet been elucidated completely. The structure of a fish tTG has been reported [44] using X-ray crystallography. In the reported structure there are no disulphide bonds and all the SH groups are free. However, the authors have noted that they used DTT during the formation of the crystal, so the information given in the paper may not be very reliable, as far as disulphide bonds are concerned.

Our results shown in Figure 3 strongly imply that guinea-pig liver tTG has disulphide bonds. We believe that the PDI reaction executed in the absence of oxygen and GSSG is a partial re-action of the entire enzymic reaction of PDI. The disulphide bonds in tTG are only simply reduced to SH groups and never re-oxidized to the original disulphides. This notion was substantiated by the experiment demonstrated in Figure 4, in which tTG whose SH groups had been alkylated with IAA prior to the experiment was used as the enzyme. It was demonstrated clearly in this figure that the disulphide(s) was (were) acting as the oxidizing equivalent during the catalysis conducted in the absence of oxidants such as GSSG and oxygen. After the reaction the disulphide(s) was (were) reduced to SH groups and they became susceptible to alkylation with IAA. When we added GSSG to the reaction mixture after the initial reaction, the enzymic reaction became very fast, probably due to the re-oxidation of the reduced disulphides. We further thought that these SH groups resulting from the PDI reaction would be re-oxidized by GSSG if it is added to the reaction mix-ture from the beginning, so that the alkylation of SH with IAA should be difficult. We did this experiment (results not shown), but the newly formed SH groups were also alkylated with IAA. We think that this lack of protection by GSSG implies that multiple numbers of disulphides are involved in the PDI reaction, and the re-oxidation of all of the newly formed SH groups by GSSG does not take place simultaneously during the cyclic reaction. The reaction mechanism would be more complicated than we assumed initially. This point will be elucidated in the near future. In fact we have determined by chemical modification with DTNB that tTG

has five or six disulphide bonds in the molecule. It remains to be elucidated in the future which of those disulphides are involved in the reaction.

In order to show that what we are looking at is really the PDI reaction, we have to be able to show the 'turnover' of the reaction, and that scrambled RNase should also be utilized as the substrate. These points were demonstrated clearly by the experiments shown in Figures 9 and 10.

One of the most important things for us to do was to establish that tTG itself, but not any contaminating proteins, really has PDI activity. We purified tTG by immunoaffinity chromatography using a monoclonal antibody against guinea-pig liver tTG. Although the purity determined by SDS/PAGE was very high, it was still possible, theoretically speaking, that some contaminating protein was the molecule with PDI activity. It is difficult to remove this kind of molecule by immunoaffinity chromatography, especially when it makes a complex with tTG. In order to circumvent this problem, we first cut out the tTG molecule from the membrane to which it was transferred from the gel of SDS/PAGE, and tested the PDI activity. We could not detect the activity there, probably due to denaturation of the protein during SDS/PAGE. We then employed gel-filtration chromatography. If the protein makes a stoichiometrical complex with tTG, the apparent mass of tTG with PDI activity should be higher than expected. In addition, the SDS/PAGE pattern of the active fraction should have bands of the protein as well as tTG, and the intensity of the bands should be fairly comparable. On the other hand, if tTG itself has PDI activity, the main peak of the chromatogram should have the expected apparent mass of tTG and PDI activity, and the SDS/PAGE pattern should be very clear with a single band of tTG. Results shown in Figure 5 give us the confidence to conclude that tTG itself has PDI activity.

The results shown in Figure 7, that rdBSA but not native BSA inhibited the tTG-mediated reactivation of rdRNase, strengthen the notion that tTG actually has PDI activity. In this experiment the concentration of the substrate rdRNase was 14.7 μM. rdBSA looks like a preferred substrate to rdRNase on a molar basis. However, the numbers of SH groups in these molecules are different: rdBSA has 35 and rdRNase has 8. If we take this into consideration, 3 μM rdBSA and 14.7 μM rdRNase become comparable in terms of number of SH groups. In fact, at this concentration rdBSA exhibited about 50% inhibition. We can then expect for rdBSA and rdRNase to be utilized equally well as the substrate. The lack of complex formation between rdBSA and rdRNase at high concentrations of rdBSA is interesting and could be an important phenomenon. This is because it suggests that SH groups of rdBSA might have been oxidized to disulphides within the molecule in the presence of SH groups in rdRNase and other molecules of rdBSA. This point will be investigated further in the near future.

Our results demonstrated clearly that the active sites for PDI and that for TGase in tTG are different from one another. The activity of PDI was not inhibited by almost complete modification of free SH groups of tTG (Figure 3). This could also mean that the free Cys might not be involved in the active site of tTG-mediated PDI activity. According to the published amino acid sequence of guinea-pig liver tTG [32], there is no consensus se-quence of CGHC in the molecule. This is in sharp contrast to the conventional PDI, which usually has CGHC as the common motif for the active site [25]. This interesting and important point should be clarified and reported in the near future. This PDI activity did not require Ca^{2+} and was not inhibited by nucleotides (Figures 12 and 13). Taken together, these results imply that tTG might be able to function as PDI but not as TGase in cytosol, where the concentrations of Ca^{2+} and nucleotides are very low

and fairly high, respectively. As the reaction catalysed by PDI is oxidation/reduction, glutathione is generally used as the cofactor. We also investigated the effect of GSSG and GSH, as shown in Figure 8. The activity supported by 10 μM GSSG was not inhibited very substantially by GSH until it was added at a 200-fold molar excess. This could mean that tTG functions as PDI in a moderately reducing environment such as cytosol, where the majority of tTG is found in cells. However, there has been no definite report as to the careful characterization of PDI in cytosol. The distribution of PDI is generally believed to be specific in the lumen of the ER, but a recent review article surveys reports of its distribution in non-ER fractions including cytosol, nucleus and cell-surface fractions [45]. The functional significance of these PDIs in these fractions remains to be elucidated.

We have determined the specific activity of tTG-mediated PDI and the commercial preparation of bovine liver PDI (Figure 11). According to the manufacturer the purity of the latter preparation is more than 95 %, as estimated by SDS/PAGE. Although our data suggest that tTG has only about 5 % activity relative to that of conventional PDI, they do not necessarily mean this tTG-mediated PDI activity is meaningless. RNase A is a typical protein to be secreted and probably is a good substrate for the conventional PDI. If we could find the right substrate for tTG-mediated PDI activity, this relative activity might be different from this value. The comparison of activities we have made between tTG and PDI (Figure 11) may also become more meaningful if we could do this experiment with the full complement of correct reductants/oxidants, chaperones and appropriate protein components. These remain to be elucidated in the future. Considering the differences in environment between the ER and cytosol, the experiment that has been done here should certainly not be taken as a meaningful physiological comparison between tTG and PDI in terms of enzymic efficacy.

Recently several papers have been published which reveal that certain PDI species have TGase activity as well. A PDI homologue protein found in the filarial parasite *Dirofilaria immitis* was reported to have both PDI and TGase activities [46]. They used a commercial preparation of bovine liver PDI and showed that it also had TGase activity. Three species of PDI were cloned from the protozoan parasite *Girardia lamblia* [47]. The expressed enzymes had TGase as well as PDI activity. They also showed that even human PDI had weak TGase activity, although there was no description about the purity and the origin of this PDI. A PDI homologue was also cloned from *Caenorhabditis elegans*, and it was shown that it also had TGase as well as PDI activity [48]. None of these works, however, attempted quantitative comparison of specific activities of PDI-mediated TGase and a proper TGase like tTG. It is difficult to tell how significant the PDI-mediated TGase activity is. None of these works showed whether these two activities were catalysed by independent active sites within one molecule. We have shown in the present work that a well-known TGase, tTG, has substantial PDI activity, probably using an independent active domain from that used for TGase. We believe that this is the first paper to report that TGase has PDI activity.

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